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SDHD Variants Do Not Constitute a Risk Factor for Developing C-Cell Hyperplasia, or Sporadic Medullary Thyroid Carcinoma

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ABSTRACT: Medullary thyroid carcinoma (MTC) is a tumor that arises from parafollicular cells of the thyroid gland. MTC can occur sporadically (75%), or as part of inherited cancer syndromes (25%). In most cases hereditary MTC evolves from preneoplastic C-cell hyperplasia (CCH), so early detection of this pathology would evidently be critical. A recent study reports that alterations in *SDHD* are responsible for familial non-*RET* CCH. We firstly studied *SDHD* in two families with hereditary non-*RET* CCH, and found no alterations related to the inheritance of this disease. We then investigated whether the H50R variant could be a risk factor in the sporadic development of MTC both in Spanish and English patients. We found no evidence that the presence of the H50R is strongly associated with the risk of sporadic MTC, though we did observe an association with age at diagnosis of MTC in Spanish H50R carriers that we did not find in English patients. Finally, we looked for evidence of CCH or any other thyroid disease in a panel of germ-line *SDH* (*B* or *D*) mutation carriers, and found none. We conclude that *SDHD* variants do not constitute a risk factor for developing CCH, or sporadic MTC.

Medullary thyroid carcinoma (MTC) is a calcitonin-secreting tumor originating in the parafollicular cells (C cells) of the thyroid gland, which represents about 5-10% of all thyroid malignancies. MTC occurs both in hereditary (25%) and sporadic (75%) clinical settings (1). In familial cases, it only occurs in familial medullary thyroid carcinoma (FMTC), or as a component of multiple endocrine neoplasia type 2A (MEN-2A) or MEN-2B (2). Although C cell hyperplasia (CCH) is a relatively common abnormality in middle-aged adults, CCH usually precedes as precursor lesion the development of MTC (3, 4). In fact, most patients with hereditary MTC firstly develop CCH (5), hence early detection of this pathology is vital in the clinical outcome of these patients.

A recent study (6) described a family with CCH as being attributable to a change in *SDHD*. This gene codes for one of the mitochondrial succinate dehydrogenase (SDH) subunits and has been found altered (as indeed have both *SDHB* and *SDHC* genes), both in paraganglioma (PGL) and pheochromocytoma (PCC) families (7-9). The authors found that the three affected members of the family had the variant c.149A>G (H50R), an alteration with controversial significance that has been related to both Merkel cell carcinomas and midgut carcinoids (10). Contrary to its apparently pathogenic character, the H50R variant has been found in 3% of two distinct control populations (11, 12). Moreover, it has been recently demonstrated that this alteration does not affect the activity of the SDHD protein (13).

The goal of this analysis was to study the involvement of *SDHD* and related genes in non-ret C cell hyperplasia, using three approaches: (a) screening these genes for abnormalities in non-ret CCH families; (b) performing an association study in MTC; and (c) investigating evidence of raised calcitonin levels in individuals carrying either the H50R variant or *SDH* mutations.

Subjects and Methods

Subjects

Informed consent was obtained from all patients. *CCH families*. We studied the presence of mutations in *SDH* genes in all affected and non affected members of two CCH families (F1 and F2), tested negative for mutations in *RET* (exons 1-20). *RET* gene segregation with the disease was studied in both families by means of haplotype analysis using intragenic SNPs located in exons 2 (rs1800858), 7 (rs1800860), 11 (rs179939), and 15 (rs1800863). We also studied *SDHD* segregation by using three SNPs (rs10789859, rs3839946, and rs7944155) chosen using PupaSNP available at http://pupasnp.bioinfo.cnio.es/ beongin and HapMap available at http://www.hapmap/org. The proband of F1 was surgically operated for MTC in 1984, and he has 12 relatives with elevated basal or pentagastrin-

provoked (PG) calcitonin levels. Total prophylactic thyroidectomy was performed on 10 of these relatives, and CCH has been confirmed in 9 of these cases by means of pathological studies (Table 1). The proband's mother in F2 was diagnosed and operated for MTC in 1982. Subsequently, four relatives showed elevated basal or PG calcitonin levels, and three of them underwent prophylactic thyroidectomy (Table 1).

Sporadic MTC patients. We studied the H50R variant both in Spanish and English patients. All Spanish (n=120) and English (n=135) MTC patients were previously tested negative for mutations in *RET* by means of sequencing of exons 10, 11, 13-16. No patients had a family history.

SDH (*B* or *D*) *mutation carriers.* Thyroid pathology was excluded in all cases by evaluating the basal calcitonin (BC) level, and in some cases by ultrasound scan (US), although CCH is difficult to exclude using these methods.

Controls. A set of 319 controls were selected from a larger group (up to 382) of unrelated and non affected individuals representative of the Spanish population. The Spanish control population was selected to be frequency matched by age and sex strata to the population of cases, with a control/case ratio equal to 3:1. Among Spanish men aged 45 to 64 however, only one control per case was available. A group of 547 anonymous individuals were used as representative of the English population. These English individuals were aged between 47 and 76 years old randomly selected from the EPIC study (14).

Methods

Amplification and sequencing analysis. Genomic DNA was extracted both from patients' and controls' blood samples following a standard method (15).

Denaturing high performance liquid chromatography (dHPLC). In order to analyze the prevalence of the H50R variant in sporadic MTC cases, DNA samples from all patients were prepared in PCR-96 plates for dHPLC analysis (Transgenomic, Cheshire, United Kingdom). We used DNA from unrelated and unaffected individuals as a control population. Exon 2 *SDHD* primers were used to genotype both control and sporadic MTC samples for H50R polymorphism using dHPLC (16). Representative samples of all defined dHPLC patterns were studied using direct sequencing to verify different genotypes.

Statistical analysis. In the Spanish population, Odds ratios (ORs) were calculated to estimate the association between H50R polymorphism and sporadic MTC, using unconditional regression analysis, adjusted for age and sex. Since the ages of diagnosis for cases with and without the studied polymorphism were substantially different (Wilcoxon text), ORs were also computed separately for two age groups, considering a cut-off equal to 45 years. Furthermore, the statistical significance of the interaction between H50R polymorphism and age was evaluated including the corresponding interaction term in the logistic model. As we did not have information on the ages of English controls, we were only able to establish the general effect of the variant using all controls, without adjusting by age.

Results and Discussion

We first studied SDH in families with hereditary non-*RET* CCH (with some cases affected with MTC), to determine whether the H50R variant, or some other SDH change, could be involved in the inheritance of this disease. We screened two CCH

families (Table 1) previously tested negative for *RET* alterations not only by a complete mutation screening, but also by means of an haplotype study. We carried out the analysis of the H50R variant, and found no change in the probands or in any of their relatives. Extended study of the remaining *SDHD* exons and of the other SDH subunits, *SDHB* and *SDHC,* similarly did not detect any alteration among these families. We also excluded both *SDHD* segregation with the disease and the presence of gross deletions in the gene in F1, by means of an haplotype study using three informative markers throughout the gene. After the molecular analysis of all SDH genes, we can conclude that the development of non-*RET* familial CCH was not attributable to the H50R variant or any other alteration of the SDHD subunit in these two families. We also suggest that the other SDH subunits are not related to the disease either.

Secondly, we adopted an approach to consider whether H50R is causally associated with CCH. Since CCH precedes MTC in most cases as a premalignant precursor, we carried out a case-control study of 120 Spanish MTC cases and 319 control individuals (Table 2). We found no evidence that the presence of H50R was associated with risk for sporadic MTC (*P*=0.261, Odds_ratio=1.68; 95% confidence interval, C.I.= [0.68-4.16]). Nevertheless, a formally significant interaction between age and the effect of the H50R polymorphism was found (*P*=0.03). The mean age at onset of our sporadic MTC was 52 years, and interestingly among H50R patient carriers, 5/8 were under 40 years of age when disease became apparent. Considering these findings, we estimated the effect of the presence of this variant in individuals aged younger than 45 years, a cut-off that corresponded to the first tertile of the age distribution among cases. A statistically significant association between the H50R variant and sporadic MTC was found (p=0.025). The effect found in the group younger than 45 years, for whom the influence of genetic risk factors could be stronger, suggested that this variant might play a role as a low penetrance gene. To examine this further, we extended the study to an English series of 135 MTC patients compared with 547 control individuals. We did not find any statistical association between the presence of this variant and the risk for sporadic MTC studying the effect of H50R in all English cases *versus* controls (Table 2). We could not perform unconditional regression analysis adjusted for age because we lacked these data, but no interaction between age and the effect of the H50R polymorphism was apparent in the English cases (observed frequencies of H50R were 1/61 and 1/71 in younger and older cases respectively). Combining data from both studies gave a crude OR estimate of 1.22 (95% C.I.= 0.52-2.63). It is likely that the association found in Spanish patients occurred randomly due to the size of our series of patients. In order to exclude the possibility that this variant was in linkage disequilibrium (LD) with another alteration with potential phenotypic effect and present only is the Spanish population, we studied further one of the SNPs used in the haplotype analysis of *SDHD* (rs3839946). This SNP was located in the promoter region of *SDHD*, and could affect the FOXD3 (transcription factor) recognition sequence. We checked for LD between these two variants among the Spanish cases, and we did not find any association between the presence of H50R and this putative functional polymorphism (data not shown).

Finally, since the H50R *SDHD* variant had been described as causative of CCH, it seemed logical to assume that other SDH variants might have similar consequences. We used plasma calcitonin measurements to test for the presence of CCH (17). We collected clinical data from 10 patients with *SDH* (*B* or *D*) mutations and 3 H50R carriers. We did not find any abnormal BC levels in the H50R carriers or amongst our SDH mutation cases. The US data did not reveal any thyroid pathology either.

In some *RET* mutation carriers, calcitonin levels are normal with C-cell disease in early stages of development, so we cannot exclude thyroid pathology entirely. Although we had no PG data (using this test in our cases with basal calcitonin levels less than 10 picograms per ml was considered unjustified because of the adverse effects), it is interesting to emphasize that none of the H50R carriers had familial antecedents of PCC or thyroid pathologies. In addition, one of the H50R carriers had inherited the variant from his mother, and it is clear that the disease transmission associated to *SDHD* mutations exclusively occurs through paternal transmission (18). Penetrance and phenotypic expressiveness of mutations in the SDH genes have not been established yet, so we cannot exclude that some pathogenic mutations lead to disease at different ages and in different locations. A recent study suggested *SDHB* mutations predisposing to early-onset kidney cancer with clinical implications for medical surveillance (19). Nevertheless, despite the fact that mutations of *SDHD* have been suggested as causing other neuroendocrine tumor development, to date there is no evidence of these genes being involved in other diseases than PGL or PCC, and the present study further excludes its involvement in CCH and MTC development.

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Table 1. Clinical data of members of families F1 and F2

Case	Birth (year)	Onset (year)	BC (pg/ml)	Thyroid histology†	
				MTC	CCH
F1					
I-1	1935	1984	-	Yes	-
II-1	1964	1998	34.7 (PG*)	-	Yes
II-2	1967	1990	102.5 (PG)	-	Yes
II-3	1971	2003	10.08	-	-
II-4	1969	2003	19.6	-	Yes
II-5	1976	-	-	-	-
II-6	1973	-	-	-	-
II-7	1978	2003	11.2	-	Yes
II-8	1988	2003	101	-	Yes
II-9	1982	2003	16.7	-	Yes
III-1	1991	-	14.7	-	-
III-2	1994	2004	16.3	-	Yes
III-3	1994	2004	17.1	-	Yes
III-4	1989	2001	10.2	-	No
III-5	1986	2003	75.3	-	Yes
F2					
I-1	1926	1982	-	Yes	-
II-1	1947	1991	118.2 (PG)	-	Yes
II-2	1960	-	-	-	-
II-3	1949	1992	101.4 (PG)	-	Yes
II-4	1953	-	-	-	-
II-5	1944	-	-	-	-
II-6	1958	-	-	-	-
II-7	1951	1992	92.1 (PG)	-	Yes
III-1	1984	2004	11	-	-
III-2	1982	-	-	-	-

*PG, pentagastrin-provoked calcitonin; BC, basal calcitonin: a normal value is less than 10 picograms per ml. † CCH was diagnosed when at least three fields (100 magnification) containing more than 50 C cells were found (20).

Table 2. Association between H50R variant and sporadic MTC in: a) Spanish population; b) English population

a

H50R	Controls	Cases	OR[1]	95% CI[1]	p-value[1]
All cases and controls					
Negative	306 (96%)	112 (94%)	1.00		
Positive	13 (4%)	8 (7%)	1.68	0.68-4.16	0.261
Younger than 45					
Negative	120 (98%)	36 (88%)	1.00		
Positive	3 (2%)	5 (12%)	5.56	1.27-24.38	0.023
Equal or older than 45					
Negative	186 (96%)	76 (96%)	1.00		
Positive	10 (5%)	3 (4%)	0.73	0.20-2.74	0.646

[1] Age (in years) and sex-adjusted estimator

b

H50R	Controls	Cases	OR[1]	95% CI	p-value[1]
All cases and controls					
Negative	532 (97%)	133 (99%)	1.00		
Positive	15 (3%)	2 (1%)	0.53	0.06-2.34	0.400
Younger than 45					
Negative		60 (98%)	1.00[1]		
Positive		1 (2%)	0.59	0.01-3.98	0.610
Equal or older than 45					
Negative		70 (99%)	1.00[1]		
Positive		1 (1%)	0.51	0.01-3.40	0.506

[1] Using all controls

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